

Identification of Suberization-associated Anionic Peroxidase as a Possible Allergenic Protein from Tomato

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A 45 kDa protein, which is recognized by IgE antibodies in sera of food-allergic patients, was purified and characterized as an allergenic protein from the tomato. The IgE-binding protein purified from tomato extract was found to be a glycoprotein with a molecular weight of approximately 45,000, an isoelectric point of 4.2, and no free N-terminal amino group. Furthermore, it was shown that the purified protein had peroxidase activity. From the amino acid sequence of a peptide fragment prepared by lysylendopeptidase digestion, the allergenic protein was identified to be the tomato suberization-associated anionic peroxidase 1 known as one of the pathogenesis-related proteins widely distributed in plants. These properties suggested the protein isolated from tomato to be a new allergenic protein in plant foodstuffs.

Key words: allergen; tomato; IgE; suberization-associated anionic peroxidase 1; pathogenesis-related protein

The tomato is a widely produced and consumed vegetable in raw and processed products. Recently, several studies have reported on the allergenicity of tomato (*Lycopersicon esculentum*) in relation to the pollenosis and oral allergy syndrome (OAS), which is an IgE-mediated type 1 allergy caused by plant-originated allergens.^{1–6} Kondo *et al.*⁷ did immunoblotting to identify the proteins as possible allergens, which are recognized by IgE antibodies in sera from patients with food allergy and OAS to tomato. Polygalacturonase 2A (46 kDa), β -fructofuranosidase (22 kDa), superoxide dismutase (18 kDa), and pectinesterase (14 kDa) were demonstrated to be the IgE-binding allergenic proteins in tomato.⁷

While the series of immune responses underlying hypersensitivity reactions in allergic patients, from the initial sensitization with allergens to the release of

chemical mediators, has been widely studied and elucidated, the common structures and physiological functions present in the allergenic proteins have not been well characterized. Current studies suggested that proteins classified into particular groups, such as pathogenesis-related proteins (PR-P), were responsible for allergic reactions.^{8–10} Many efforts have been made to resolve the relationship between the structure or function of proteins and their ability to induce IgE production in an immune system. In order to identify common features of allergenic proteins, we have isolated and identified the allergenic proteins from various plant foodstuffs as the proteins recognised by patient's IgE antibodies. Although these findings obtained by the *in vitro* tests often conflict with the signs derived from the *in vivo* tests such as the skin prick test or their relevance to the patient's manifestations, the more precise *in vitro* examinations, such as immunoblotting analyses of proteins binding with patient's IgE antibodies, will be still important to get information about the unknown causative allergens in diverse plant food items. Accordingly, in this series of experiments we considered that the proteins recognized by specific IgE antibodies of patient's sera are the strong candidates for allergens inducing production of IgE antibodies.

This study describes the purification and characterization of the 45 kDa IgE-binding protein in the tomato, which was recognized by the sera of the patients suspected to suffer from food allergies and diagnosed to be atopic dermatitis, as a new possible allergenic protein in the tomato.

Materials and Methods

Materials. Fresh tomatoes (*Lycopersicon esculentum*) used in this study were obtained from a local market at Uji, Kyoto. TSK gel SuperQ Toyopearl

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Abbreviations: IgE, Immunoglobulin E; OAS, oral allergy syndrome; PR-P, pathogenesis-related proteins; HPLC, high-pressure liquid chromatography; IEF, isoelectric focusing; ECL, enhanced chemiluminescence; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2D, two dimensional; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline

650M, a Butyl Toyopearlpack 650M HPLC column, and a TSK gel SuperSW3000 HPLC column were purchased from TOSOH (Tokyo, Japan). A Centricon-30 and Microcon-30 were acquired from Amicon (Beverly, MA, USA). Immobiline IEF gels (pH 3–10), PD-10 columns, Rainbow protein markers, Coomassie brilliant blue R350, an ECL western blotting kit, and an ECL glycoprotein detection system were obtained from Amersham Biosciences Corp (Piscataway, NJ, USA). A protein assay kit, nitrocellulose membranes, and a copper stain kit were obtained from Bio-Rad (Richmond, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE was acquired from KPL (Gaithersburg, MD, USA). Gel filtration molecular weight markers were obtained from Sigma (Saint Louis, Missouri, USA).

Patient sera. Serum samples were selected from 361 patients with atopic dermatitis (mean age 6.1 years old, ranging from 3 months to 23 years) who were asked to donate blood samples for testing at hospitals in the Shikoku area after complete informed consent.¹¹⁾ According to case histories, the suspected allergenic food components, such as egg white, soybean, wheat, milk, and rice were recorded, but no strict *in vivo* diagnostic tests, such as a skin prick test or double-blind placebo-controlled food challenges, were done because the subjects received treatment as outpatients only. Randomly selected patient sera were screened by immunoblotting techniques in this study. An additional pooled serum sample obtained from 5 non-allergic healthy individuals served as a negative control.

Protein assay. Protein concentrations were measured by the Bradford method¹²⁾ with a protein assay kit (Bio-Rad) using bovine γ -globulin as a standard.

Preparation of tomato extract. One and one-tenth kg of fresh tomato were cut off the calyx and homogenized in 30 mM Tris-HCl (pH 8.0) using a Polytron homogenizer. The homogenate was filtered through three layers of gauze, and then the filtrate was centrifuged at $15,000 \times g$ for 20 min at 4°C to remove fiber and cell debris. The proteins in the supernatant were recovered by precipitation with 75% saturated ammonium sulfate. The precipitate was collected and dissolved in 30 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (Buffer A), followed by dialysis against the same buffer for 20 h at 4°C. The extract was stored at -28°C until use.

Purification of a tomato 45 kDa allergen. Tomato extract was put on a TSK gel SuperQ Toyopearl 650M (column volume 50 ml) previously equilibrated with Buffer A and the absorbed proteins were eluted stepwise with 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl in

Buffer A. The 45 kDa protein, which was recognized by the patient's sera, appeared in the 0.1 M NaCl fraction of Buffer A. The protein was recovered by precipitation with 75% saturated ammonium sulfate. The precipitate was dissolved in Buffer A, and then put on a Butyl Toyopearlpack 650M HPLC column (22 mm $\times$ 20 cm), which was previously equilibrated with Buffer A containing 1 M ammonium sulfate. Elution was done with a linear gradient from 1.0 to 0 M ammonium sulfate in Buffer A at a flow rate of 0.5 ml/min. The fractions containing the 45 kDa IgE-binding protein were pooled and dialyzed against Buffer A for 20 h at 4°C. The protein (2.5 mg) which was concentrated by Centricon-30 was analyzed by HPLC with a SuperSW3000 gel filtration column (4.6 mm $\times$ 30 cm) using Buffer A containing 0.3 M NaCl at a flow rate of 0.2 ml/min. Fractions containing the 45 kDa protein recognized by patient's sera were pooled and concentrated by Microcon-30.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 10% polyacrylamide gels according by the method of Laemmli.¹³⁾ For immunoblotting analyses, the proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane under semidry conditions.¹⁴⁾ The transferred membrane was incubated in 10 mM sodium phosphate buffered saline (PBS, pH 7.5) containing 0.1% Tween-20 and 5% skim milk for blocking. The membrane was incubated overnight with patient sera diluted 1:10 in PBS at 4°C. Bound IgE antibodies were detected using HRP-conjugated goat anti-human IgE and an ECL-Western blotting kit. Proteins on the gel were stained with Coomassie brilliant blue R350.

Two-dimensional (2D) PAGE. For the first dimension, isoelectric focusing (IEF) was done using Immobiline precast gel strips (pH 3–10), according to the manufacturer's instructions under non-reducing conditions, and for the second dimension, SDS-PAGE was done under reducing conditions.

Analysis of N-terminal amino acid sequence. The partially purified 45 kDa allergen was put through 2D PAGE as described above and separated proteins were detected with a copper staining kit. The 45 kDa protein with IgE-binding activity was digested directly on the gel with lysylendopeptidase (5.625×10^{-3} AU/spot of protein) in 50 mM Tris-HCl buffer (pH 9) for 3 h at 37°C. The digested peptides were separated by SDS-PAGE on 12.5% polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes, and then stained with 0.1% Ponceau S containing 1% acetic acid. The bands corresponding to the digested peptide were cut out and their N-terminal amino acid sequences were found,

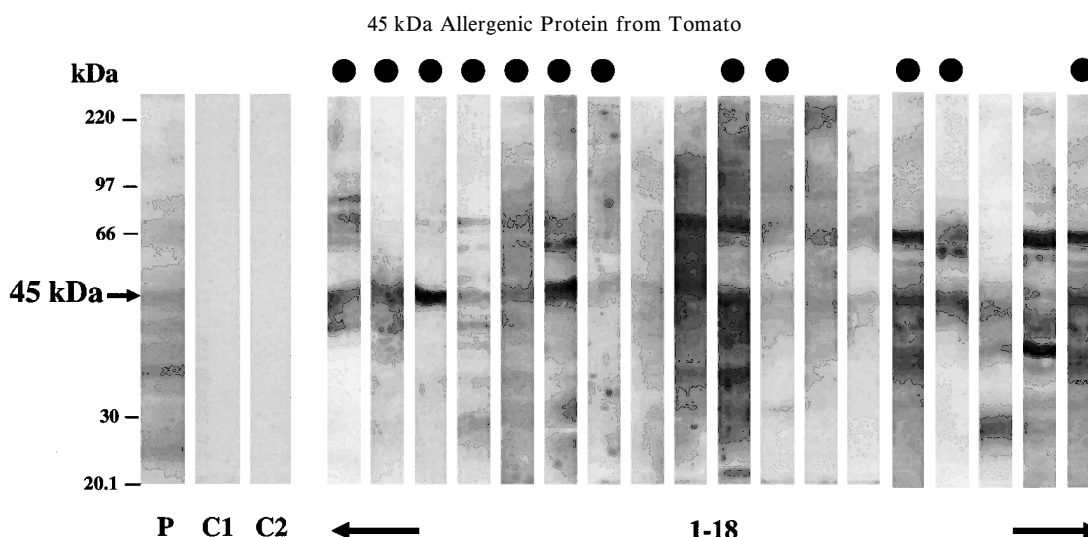


Fig. 1. IgE-Binding Proteins in Tomato Extract.

The ECL fluorograms of the immunoblots obtained from 18 randomly selected patients' sera (see "Materials and Methods"). The lanes indicated by closed circles represent IgE antibodies from the patients' sera recognizing the tomato protein with a molecular mass of about 45 kDa. Molecular masses of the standard proteins are indicated on the left. Lane P represents tomato proteins stained with Coomassie brilliant blue R350. Lane C1 and C2 show immunoblots with pooled serum from non-allergic healthy volunteers and buffer instead of serum as controls. Lane 1–18 represent immunoblots with serum from food-allergic patients with atopic dermatitis.

using an Applied Biosystems 492 protein sequencer (Perkin Elmer, Foster City, CA, USA).

Peroxidase assay. Five μ l of each fraction (no. 6–19) from the SuperSW3000 gel filtration column was mixed with 100 μ l of an ECL western blotting kit in a White Cliniplate (Labsystems, Helsinki, Finland). The peroxidase activity of each fraction was detected by luminescence using Wallac ARVO (Perkin Elmer, Foster City, CA, USA).

Detection of carbohydrate moiety. To identify the carbohydrate moieties, the pooled fraction containing the 45 kDa allergenic protein was blotted onto nitrocellulose membranes and detected by an ECL glycoprotein detection system by the manufacturer's protocol.

Results

Screening of IgE-binding proteins

As a first step, we examined which proteins in tomato extract were recognized by the sera from the allergic patients. A protein with a molecular mass of about 45 kDa was shown to be strongly recognized by patients' sera in about 67% of the allergic patients (12 serum samples from 18 patients). The immunoblot profiles of patients 1–18 are represented in Fig. 1. Furthermore, proteins with molecular masses of about 42 and 62 kDa were also found to bind with IgE from patients' sera (Fig. 1).

Purification of 45 kDa allergenic protein

The 45 kDa allergenic protein in tomato extract was chromatographed on a superQ anion exchange

column and fractionated in the 0.1 M NaCl fraction of Buffer A. The allergen absorbed on a butyl hydrophobic interaction column was eluted with a linear gradient of 1.0–0 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer A. The fractions containing the 45 kDa IgE-binding protein were mainly eluted in the fractions of 1.0 to 0.8 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer A. Finally, the protein was further chromatographed by HPLC with a SuperSW3000 gel filtration column and the 45 kDa allergenic protein was partially purified as a single peak (Fig. 2). On the 2D PAGE of pooled fractions, it was confirmed that the major protein spot on the membrane, with a *pI* of 4.2 (indicated by an arrow), corresponded to the 45 kDa tomato allergen recognized by patients' sera (Fig. 3A, B). This preparation of the 45 kDa allergen was studied in further experiments.

Amino acid sequence of a peptide fragment of 45 kDa allergen

As no free N-terminal amino group was detected in the purified protein, the tomato 45 kDa allergen was digested with lysylendopeptidase to obtain internal peptide fragments. The N-terminal amino acid sequence of a digested peptide was found to be LGGQTYSVALLG-, which closely matched the partial amino acid sequence of tomato suberization-associated anionic peroxidase 1 (SWISS Protein database accession no. P15003) by a computer homology search using FASTA program. Alignments of partial amino acid sequences of tomato 45 kDa allergen and related peroxidases are shown in Fig. 4.

Peroxidase activity of tomato 45 kDa allergen

The sequence of tomato 45 kDa allergen was shown to be similar to suberization-associated anionic peroxidase 1. We examined whether this allergenic protein had peroxidase activity. The fractions containing 45 kDa allergen (fractions 11–19) from the SuperSW3000 gel filtration column chromatography were put through a peroxidase assay under the conditions described in the materials and methods section. Figure 5A shows that fractions 11–19 had peroxidase activity.

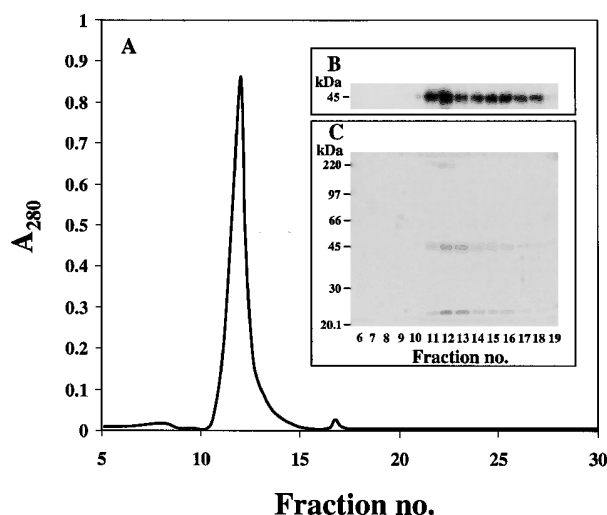


Fig. 2. Purification of the Tomato 45 kDa Allergen.

A, Chromatogram on SuperSW3000 column; B, Immunoblotting profiles of eluted fractions with patients' sera; C, SDS-PAGE pattern of eluted fractions (proteins stained with Coomassie brilliant blue R350). Experimental details are described in "Materials and Methods".

Carbohydrate moieties of tomato 45 kDa allergen

According to the amino acid sequence deduced from the cDNA of suberization-associated anionic peroxidase 1, this protein was shown to have seven consensus asparagine N-linked glycosylation sites (-N-X-S(T)-). The positive reaction against a glycoprotein detection system demonstrated that the tomato 45 kDa allergen is in fact a glycoprotein (Fig. 6).

Discussion

Food-specific IgE antibodies bind to high-affinity FcεR1 receptors on mast cells, basophils, macrophages, monocytes, lymphocytes, eosinophils, and platelets. When food allergens pass through mucosal barriers and reach IgE antibodies on mast cells or basophils, chemical mediators such as histamine released from these cells elicit symptoms of immediate hypersensitivity (type 1 IgE mediated reaction).¹⁵⁾ In this study, a protein from tomato with a molecular mass of about 45 kDa, which binds to IgE from sera of patients with food allergy, was identified as a candidate of allergenic protein.

Using several different types of chromatographic procedures and 2D PAGE, the allergenic protein was partially purified (Fig. 2, 3). Furthermore, the apparent molecular mass of the purified protein obtained from the SuperSW3000 gel filtration column was estimated to be 45 kDa based on the calibration curve (Fig. 5B). When the N-terminal amino acid sequence of the isolated protein was measured using a protein sequencer, it was seen that the N-terminal amino group was blocked. Accordingly, the tomato 45 kDa

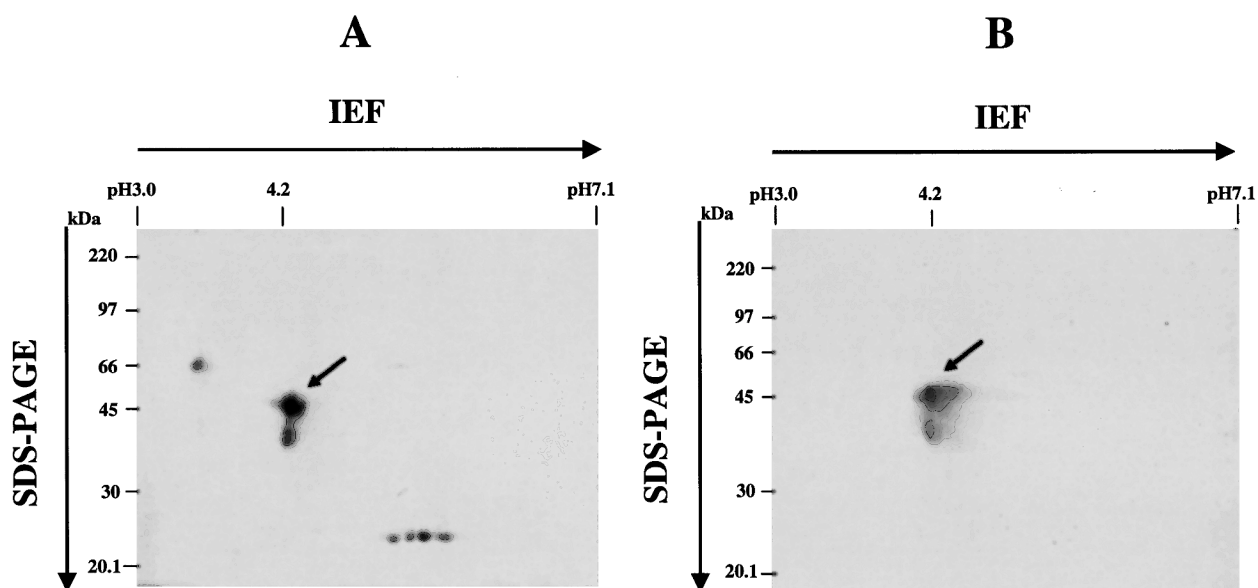


Fig. 3. 2D PAGE of the Tomato Allergen.

The pooled fractions of Fig. 1 (fractions 11–19) were concentrated and put through 2D PAGE as described in "Materials and Methods". A, Staining with Coomassie brilliant blue R350; B, Immunoblot using patients' sera. Arrows indicates 45 kDa allergen.

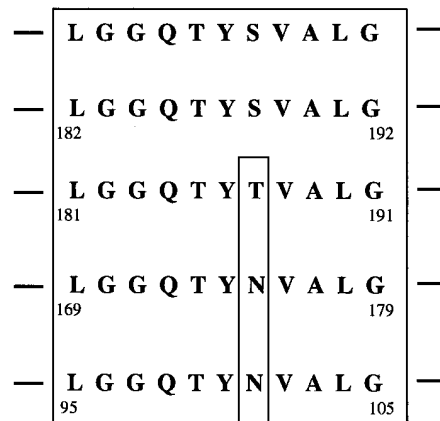
Tomato 45 kDa allergen**Suberization-associated anionic peroxidase 1**
(*Lycopersicon esculentum*, tomato)**Suberization-associated anionic peroxidase 2**
(*Lycopersicon esculentum*, tomato)**Suberization-associated anionic peroxidase**
(*Solanum tuberosum*, potato)**Petunia hybrida anionic peroxidase**
(*Petunia X hybrida*, petunia)

Fig. 4. Sequence Similarity of Tomato 45 kDa Allergen to Suberization-associated Anionic Peroxidase 1 and Related Peroxidases. Boxed portion represents the homologous amino acid residues among the five sequences.

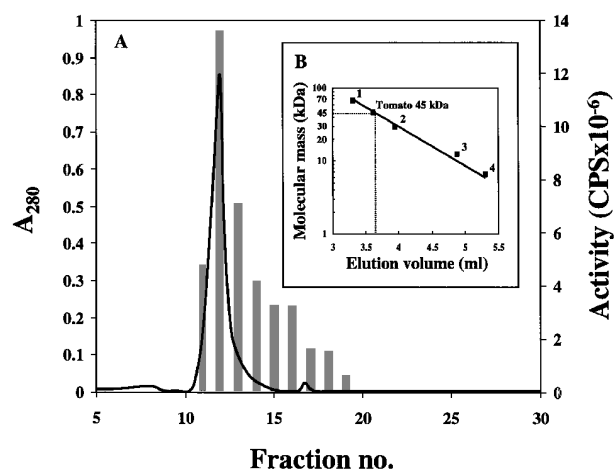


Fig. 5. Peroxidase Activity of Tomato 45 kDa Allergen.

A, The eluted fractions obtained from the SuperSW3000 column were assayed for peroxidase activity as described in "Materials and Methods". Vertical bar, peroxidase activity; Line, absorbance at 280 nm. B, Molecular mass estimation of the tomato 45 kDa allergen by gel filtration on a SuperSW3000 column. The markers used: 1, bovine serum albumin (66 kDa); 2, carbonic anhydrase (29 kDa); 3, cytochrome c (12.4 kDa); 4, apoferritin (6.5 kDa).

allergenic protein was first hydrolyzed by lysylendopeptidase, and then the sequence of the peptide fragments released were analyzed. The estimated sequence was shown to be identical with the partial sequence of tomato suberization-associated anionic peroxidase 1,¹⁶ and not to be a 46 kDa polygalacturonase 2A⁷ which was considered to migrate into a similar position on SDS-PAGE to a 45 kDa allergenic protein. This enzyme was thought to be involved in the polymerization of phenolic monomers, which generate the aromatic matrix of suberin and function in the defense mechanism against stress and pathogens.¹⁷ Sanchez *et al.*¹⁸ had purified and characterized a 36 kDa allergen from wheat, which was detected by immunoblots using sera from patients with baker's asthma, and identified this allergen as a seed-

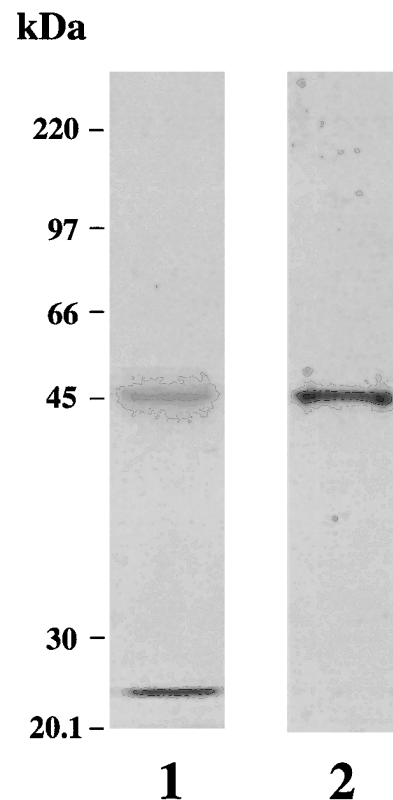


Fig. 6. Carbohydrate Moieties of Tomato 45 kDa Allergen.

The purified tomato 45 kDa allergen was put through SDS-PAGE and western blotting as described in "Materials and Methods". Molecular masses of the standard proteins are indicated on the left; 1, Protein stained with Coomassie Brilliant blue R350; 2, Glycoprotein stained with ECL glycoprotein detection system.

specific peroxidase. Interestingly, many peroxidases are known to be PR-P and also seem to be important food allergens associated with pollenosis in the environment.

Plant asparagine N-linked oligomannose glycan moieties containing β -1,2 xylose and/or α -1,3 fucose branches and similar insect glycoproteins have been

reported to be recognized by IgE antibodies from allergic patients.¹⁹ The amino acid sequence of this allergen was shown to have seven consensus N-glycosylation sites and to have carbohydrate moieties (Fig. 6). Also, the molecular mass of tomato 45 kDa estimated by gel-filtration chromatography suggested that this protein bears several carbohydrate moieties, as the mass deduced from the cDNA was estimated to be 30.6 kDa.¹⁶ However, whether the sugar moieties are a functional epitope or not still remains to be found. The relationship between recognition of a sugar moiety by the patients' sera and elicitation of clinical symptoms is currently under investigation.

Acknowledgments

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